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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Tamoxifen is effective for the prevention and treatment of estrogen-dependent breast cancers, but is associated with an increased incidence of endometrial tumors. We completed the first aim of this proposal to solve the crystal structure of the estrogen receptor alpha ligand-binding domain (ERαLBD) bound to the structurally similar compound GW5638, which has therapeutic potential and does not stimulate the uterus. Like tamoxifen, GW5638 relocates the carboxy-terminal helix (H12) to the known coactivator-docking site in the ERα LBD. However, GW5638 repositions residues in H12 through specific contacts with the N-terminus of this helix. In contrast to tamoxifen, the resulting increase in exposed hydrophobic surface of ERα LBD correlates with a significant degradation of ERα in MCF-7 cells. Thus, the GW5638-ERa LBD structure reveals a unique mode of SERM-mediated ER antagonism, in which the stability of ERα is decreased through an altered position of H12. This dual mechanism of antagonism may explain why GW5638 can inhibit tamoxifen-resistant breast tumors. In addition, difficulties encountered with experiments under aim 2 and 3 were addressed along with alternative approaches proposed in this report.

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Table of Contents

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INTRODUCTION

Breast cancers affect one in eight women in the United States. Despite the fact that estrogens are beneficial in various contexts including cognition, protective roles in the cardiovascular system and the control of reproductive functions, estrogen use has also been implicated as a risk factor in breast and uterine cancers. This suggests that a great degree of flexibility to control unwanted side effects would be desirable to target ER for breast cancer treatment and prevention (Couse and Korach, 1999; Hunt, 1994; McGuire, 1978). Accordingly, endocrine therapy is the standard care for most women with hormone-dependent tumors in adjuvant and metastatic settings.

Selective estrogen receptor modulators (SERMs) with mixed characteristics as agonists or antagonists in a tissue-specific manner have been designed to bind ER and elicit distinct pharmacological profiles. These molecules behave like estrogen in bone and cardiovascular system but block its action in the mammary tissues. The mixed agonist/antagonist properties of SERMs and relatively mild side effects explain why drugs like tamoxifen and raloxifene are used for the treatment and prevention of breast cancer and for the prevention of osteoporosis, respectively. Tamoxifen is the standard endocrine treatment for ERa-positive primary and metastatic breast cancers. Unfortunately, most of these cancers become resistant within 2-5 years and the risk of uterine cancer increases in women who take tamoxifen. Available pharmacological data showed similar activities for raloxifene, but data on the activity of raloxifene in patients with advanced disease are limited (Buzdar et al., 1988; Gottardis and Jordan, 1987; Poulin et al., 1989). The commonly used second-line endocrine therapy for tamoxifen-refractory tumors includes aromatase inhibitors, gonadotrophin releasing hormone agonists or the pure ER antagonist ICI (Fulvestrant, Faslodex). However, none of these agents has the beneficial agonist activities associated with tamoxifen.

GW5638 is a novel SERM that differs from tamoxifen in that the dimethylaminoethyoxy group is replaced by an acrylate side chain (Willson et al., 1994). This compound exhibits beneficial estrogenic properties but unlike tamoxifen, it is a more potent antagonist in breast cancer cells and has no uterotrophic behavior. Because tamoxifen-resistant breast cancers are not cross-resistant to GW5638, this SERM has significant potential as a therapeutic agent. GW5638 and its 4-hydroxy metabolite (GW7604) can induce a unique conformational change in ER that is recognized by synthetic peptides selected by phage display. These peptides recognize GW5638/GW7604-ER complexes but not tamoxifen-ER or other ligand-bound ER complexes, indicating that conformational changes elicited by GW5638 and tamoxifen are different (Connor et al., 2001). To better understand the pharmacology of GW5638, I proposed (1) to determine the structure of human ER/-LBD in complex with GW5638 (2) to determine

the structure of ER□/□-LBD/GW5638 associated with GW 7□-16 and/or other CoRNR box peptides. In addition, we also propose to determine the structure of C through E domains of human ER□/□ and to characterize the interactions among these functional domains, which may have important pharmacological significance in biology and drug design. Due to some technical difficulties while performing the original specific aims (2) and (3), I have revised my proposal to include alternative approaches in my last annual report. As I have completed my PhD studies in Jun, 2005, I will no longer pursue aim (3) in this proposal.

BODY

AIM 1: To determine the structure of human ER/-LBD in complex with GW5638 and to compare the conformational changes in ER/-LBD elicited by tamoxifen and GW5638

I have accomplished this specific aim, which includes (1) generating and purifying GW-ER LBD for biochemical analysis and crystallization; (2) obtaining single, diffraction-quality crystals for x-ray analysis; (3) determining the x-ray structure of GW-ER LBD and depositing the structure to the protein data bank.

The structure of ER LBD bound to GW5638 was solved by molecular replacement with a modified raloxifene-ER LBD structure to a resolution of 2.7 Å with an R factor of 0.208 and free R factor of 0.236 (Table 1, Appendix 1). The refined structure reveals clear electron density for GW5638 (Figure 1C, Appendix1). Like other antagonist-bound ER structures, GW-ER LBD folds into a canonical three-layered sandwich of twelve -helices. GW5638 lies in an orientation similar to OHT or RAL inside the ligand-binding pocket (Figure 1D, Appendix 1). Unexpectedly, the carboxyl groups between GW5638 and Asp 351 form a hydrogen bond at the crystallization pH of 5.6 (Figure 2A), rather than repelling each other as predicted from molecular modeling studies of the 4-hydroxy metabolite GW7604 (Bentrem et al. 2001). It is likely that the hydrophobic environment of the pocket results in protonation of the weakly acidic acrylate, allowing for hydrogen bond formation with Asp351 (Urry et al., 1992). The acrylate side chain of GW5638 induces an unexpected conformation in H12, the C-terminal "activation function-2" (AF2) helix, which has not been observed in other antagonist-bound NR LBD structures. The electron density in this region was clearly revealed and the 2Fo-Fc map was shown in Figure 1C, right, Appendix 1. In the OHT and RAL ER LBD structures, H12 binds to and occludes the coactivator-binding site by mimicking the hydrophobic interactions of coactivator NR box LXXLL motifs with the recognition groove formed by H3, H4 and H5 (Brzozowski et al., 1997; Shiau et al., 1998). This orientation of H12 is partially displaced from the hydrophobic cleft in the GW-ER complex, which can be attributed to the water-mediated hydrogen bonds that link one of the carboxyl oxygen atoms in GW5638 to the backbone NH group at the N-terminus of H12 (Figure 2C, Appendix 1). It is noteworthy that this ligand-protein interaction is very similar in every monomer in the asymmetric unit, as the rmsd values between main chains and side chains among three monomers are less than 1 Å.

Leu 536 mediates the N-terminal capping of H12, which is initiated by the water-mediated hydrogen bonds between the GW 5638 acrylate carboxyl group, Leu 536 and Tyr 537, bringing the N-terminus of H12 and the loop connecting H11 and H12 (L11-12) 6-7Å closer to the ligand (Figure 3, Appendix 1). Consequently, H12 relocates ~10° away from H11, compared to the OHT and raloxifene complexes, resulting in a ~50° difference in orientation for H12 in the

GW-ERα and OHT-ERα structures. This displacement disrupts the hydrophobic interactions between the AF-2 cleft and the H12 LLEML residues seen in the OHT-ER structure. In addition, a series of hydrogen bonds between H3/H5 and H12 in the OHT-ER structure are lost in the GW-ER structure (Figure 4, Appendix 1). Significantly, this unusual orientation induces a repositioning of hydrophobic residues in H12 (Leu-536, Leu-539, Leu-540 and Met-543), such that the side chains of these residues are no longer buried in the hydrophobic core but become solvent exposed on the protein exterior (Figure 3B, Appendix 1). Thus, GW5638 causes a nearly 27% increase in the exposed hydrophobic surface of H12 compared to the OHT-ERα structure. This surface is stabilized in part by contact with a neighboring LBD molecule in the crystal lattice.

I have also performed surface hydrophobicity of various ERα LBD complexes using bis-ANS probe. Endogenous ERα stability upon different ligand treatment was also monitored to confirm the structural information. In contrast to tamoxifen, the resulting increase in exposed hydrophobic surface of ERα LBD correlates with a significant degradation of ERα in MCF-7 cells (Figure 5 in Appendix 1), which may account for the ability of GW5638 to inhibit tamoxifen resistant MCF-7 breast tumor explants.

In short, the acrylate side chain of GW5638/7604, a tamoxifen analog, interacts with Leu-536 and Tyr-537 of the ERα LBD to induce capping of H12 and an unexpected conformation for this AF2 molecular switch. The resulting increase in ERα surface hydrophobicity correlates with a decrease in receptor stability, in sharp contrast to the increased stability observed for OHT-ERα. Notably, the ERα was significantly stabilized in the presence of GW7604 by replacement of Leu-536, Leu-539 and Leu-540 with Gln, which should reduce the surface hydrophobicity of H12. Thus, the GW5638-ERα structure adds another level of complexity to the observed conformational flexibility of H12 by showing that it not only controls the recruitment of cofactors but also influences the stability of ERα. Overall, our data suggest that antagonist-mediated increases in surface hydrophobicity can contribute to ERα instability.

The coordinates of this structure have been deposited in the Protein Data Bank (PDB ID: 1R5K). The technical details are given in the experimental procedure of Appendix 1.

AIM 2: To determine the structure of ER/ LBD/GW5638 associated with GW 7-16 and/or other CoRNR box peptides and to identify the specific interactions between GW5368 and ER that facilitate corepressor docking

The technical hurdles to obtain diffractable complex crystals as well substitutive approaches were detailed in my last annual report. Alternatively, we mapped the peptide-binding site by modeling and tested the predictions by mammalian two-hybrid assay coupled with mutagenesis. Based on the solved GW-ER structure and the sequence homology between GW-selective peptides and CoRNR box motif (Perissi et al., 1999), we proposed that GW-selective peptides bind to the putative CoRNR-binding region in the ER LBD, which partially overlaps with the coactivator-binding site in the AF-2 cleft.

A few mutations that are known to enhance or reduce the interaction of TR or RXR with NCoR/SMRT (Hu and Lazar, 1999) were introduced into ER at equivalent positions (Figure 6A, Appendix 1). ER-peptide interaction was determined by the ability of wild-type or mutant ER-VP16 to initiate transcription from the Gal4-responsive reporter in the presence of the GW compound. Deletion of H12 (ER 537X or ER 538X), which was previously shown to increase NCoR binding to ER (Webb et al., 2003), enhanced the binding of GW-selective peptides to GW-ER LBD (Figure 6B, Appendix 1). Although the L372R and V376R mutations did not have an overall effect on different GW-peptide binding, mutations L379R, at the base of H5, and K362A, at the H3/H4 boundary, significantly reduced all the interactions between ER and peptides (Figure 6C-D, Appendix 1). L379R is of interest because this residue was shown to be critical for NCoR recruitment to ER (Webb et al., 2003). In the GW-ER LBD crystal structure, H12 is partially displaced from the AF2 cleft, which may facilitate corepressor competition with H12 in the presence of GW5638/7604 compared to tamoxifen. The LBD structures correlate well with fluorescence-microsphere-binding data (Iannone et al., 2004), which showed that GW7604 and other compounds with the same acrylate or carboxylate side-chain are more effective recruiters of corepressor-like peptides to ER. It is possible that the GW-selective peptides are affinity-optimized versions of the CoRNR box peptide motif for GW-ER.

Transcriptional regulation by ER is a complex process that involves the participation of coactivators and corepressors. While the interaction of coactivators with ER is well established, the interaction and importance of corepressors is less clear (Dobrzycka et al., 2003). ER is different from other NRs, such as retinoic acid receptor (RAR) and thyroid receptor hormone (TR), because it does not appear to actively repress transcription in the absence of ligand (Chen and Evans, 1995; Dobrzycka et al., 2003). However, evidence suggests that antagonist-mediated inhibition of ER not only blocks coactivator recruitment but also facilitates the recruitment of

corepressors to form an actively repressed ER complex (Cottone et al., 2001; Dobrzycka et al., 2003; Shang and Brown, 2002; Webb et al., 2003).

Most of the estrogen responsive breast cancers that are normally growth inhibited by tamoxifen therapy, become resistant to, and even stimulated by tamoxifen. Evidence has shown that this may due to a reduction in corepressor protein levels in breast cancer cells that have been chronically exposed to tamoxifen (Lavinsky et al., 1998). Our data indicated that drugs with acrylate or carboxylate side-chain may be more effective to recruit corepressor-(like) proteins, which could account for in part why tamoxifen-resistant cancers respond to GW5638.

In the absence of an ER-corepressor structure, our structure data suggest how corepressor-like peptides might compete with the partially displaced H12 for the AF-2 cleft in the GW-ER structure. Our mammalian-two hybrid data further support the structural information in that deletion of H12 as well as two ER mutations L379R and K362A significantly alter the binding of GW-specific peptides. Notably, these peptides were obtained in the absence of any structural information. The fact that some of these peptides are homologous to the CoRNR box motif suggests that the ER conformation induced by GW5638 favors the recruitment of corepressor-like proteins, implying that corepressors could play a critical role in the pharmocology of GW5638.

The technical details of performing mutagenesis and mammalian two-hybrid assay are given in the experimental procedure of Appendix 1.

AIM 3: To determine the structure of C through E domains of human ER/ and to characterize the interactions among these functional domains, which may have important pharmacological significance in biology and drug design

I have attempted to express C through E domains of human ER/ but it is problematic to purify the full-length of this region for further biochemical analysis and crystallization.

Because I have completed my PhD studies in June, 2005, I will no longer pursue aim 3 of this proposal.

KEY RESEARCH ACCOMPLISHMENTS

During the training period, I have crystallized, determined and refined the atomic structure of ER LBD in complex with GW5638 and revealed the mechanism that may account for the ability of GW5638 to inhibit tamoxifen-resistant breast tumor explants. I have also accomplished the biochemical as well as functional characterizations with regard to GW-ER.

REPORTABLE OUTCOMES

1. The structure of GW-ER LBD was deposited to the protein data bank (PDB ID: 1R5K)
2. The structure of GW-ER along with other functional characterizations has been published in Molecule Cell, vol 18, 413-424, 2005 (please refer to Appendix 1).
3. The structural study of GW-ER LBD was presented as a poster at the Nuclear Receptor Keystone Symposia (02/28/04 – 03/04/04) supported by Keystone Symposia, held at Keystone Resort, Keystone, CO (please refer to Appendix 2 for the abstract).
4. The discoveries accomplished in the training period were presented as a poster at Era of Hope 2005 - Department of Defense Breast Cancer Research Program Meeting (06/08/05 – 06/11/05) hosted by U.S. Army Medical Research and Materiel Command, held at Philadelphia, PA (please refer to Appendix 3 for the abstract).
5. PhD thesis “Structural and Functional Characterization of Human Estrogen Receptor alpha Ligand-Binding Domain In Complex with GW5638”.

CONCLUSIONS

In summary, the GW5638-ER LBD structure and supporting data may explain why GW5638/7604 is an effective inhibitor of tamoxifen-resistant MCF-7 tumor explants. GW5638/7604 shares some of the HRT benefits of tamoxifen but acts as a more potent antagonist in the breast and does not stimulate the uterus. The data presented here show that the distinct pharmacologies of tamoxifen and GW5638 are due, at least in part, to subtle changes in the respective ER AF2 conformations. These data also suggest that an acidic side chain may be a useful substitute for drug design on the triphenylethylene scaffold. In addition to preventing coactivator recruitment by occlusion of the AF2 cleft, similar to other SERMs, GW5638/7604 also destabilizes ER, although less so than the more potent ER antagonist ICI 164,380/182,780. Therefore GW5638/7604 belongs to a class of molecules that has mixed functions (SERM/SERD) (McDonnell, 2005). SERMs function either as agonists or antagonists, depending on the coregulator context. SERDs (selective estrogen receptor down-regulators) like

ICI and ZK-703 (Hoffmann et al., 2004), act as more potent antagonists by inducing receptor destabilization. If proven to be effective in clinical trials, molecules like GW5638/7604 could be used as a second-line therapy for patients whose breast cancers have become tamoxifen-resistant. Because no single endocrine agent is likely to prevent recurrent disease in ER-positive breast tumors, there continues to be a need for novel agents that differentially target estrogen/ER-signaling pathways.

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Structural Basis for an Unexpected Mode of SERM-Mediated ER Antagonism

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Summary

Tamoxifen is effective for the prevention and treatment of estrogen-dependent breast cancers, but is associated with an increased incidence of endometrial tumors. We report the crystal structure of the estrogen receptor α (ERα) ligand binding domain (LBD) bound to the structurally similar compound GW5638, which has therapeutic potential and does not stimulate the uterus. Like tamoxifen, GW5638 relocates the carboxy-terminal helix (H12) to the known coactivator-docking site in the ERα LBD. However, GW5638 repositions residues in H12 through specific contacts with the N terminus of this helix. In contrast to tamoxifen, the resulting increase in exposed hydrophobic surface of ERα LBD correlates with a significant destabilization of ERα in MCF-7 cells. Thus, the GW5638-ERα LBD structure reveals an unexpected mode of SERM-mediated ER antagonism, in which the stability of ERα is decreased through an altered position of H12. This dual mechanism of antagonism may explain why GW5638 can inhibit tamoxifen-resistant breast tumors.

Introduction

Breast cancers affect one in eight women in the United States. Many of these cancers respond to hormonal therapy and the presence of human estrogen receptor-α (ERα) is associated with a more favorable response and short-term prognosis (Hunt, 1994). ERα is a ligand-activated transcription factor that has important functions in many tissues and plays a critical role in the etiology of breast cancer (Couse and Korach, 1999; Hunt, 1994; McGuire, 1978). Because ERα is an important target for breast cancer treatment and prevention, numerous molecules have been designed to bind ERα and elicit distinct pharmacological profiles. Tamoxifen or 4-hydroxytamoxifen (OHT) is part of a growing family of molecules called selective estrogen receptor modulators (SERMs) that can behave as agonists or antagonists in different tissue and environmental contexts. It has been widely used for breast cancer treatment and shows considerable potential as a preventive agent. Tamoxifen has protective benefits in bone and cardiovascular tissues as an ERα agonist and displays antagonistic activity in most ER-positive breast tumors. Unfortunately, advanced breast cancers that initially respond well to tamoxifen eventually become refractory to this compound. Its uterotrophic activity also restricts its utility in a prevention setting (Graham et al., 2000; McDonnell et al., 2002; Ravdin et al., 1992).

GW5638, 3-[4-(1,2-Diphenylbut-1-enyl]phenylacrylic acid), is a SERM with clinical potential that was identified in a screen for compounds that are mechanistically distinct from tamoxifen and raloxifene (Willson et al., 1994). In contrast to tamoxifen, the dimethylaminoethyoxy group is replaced by an acrylate side chain (Figure 1A). This compound exhibits beneficial estrogenic properties, but unlike tamoxifen, it is a more potent antagonist in breast cancer cells and has no uterotrophic behavior (Willson et al., 1997). Because tamoxifen-resistant breast cancers are not cross resistant to GW5638, this SERM has significant potential as a therapeutic agent. GW5638 and its 4-hydroxy metabolite (GW7604) can induce a unique conformational change in ERα that is recognized by synthetic peptides (Figure 1B) selected by phage display (Connor et al., 2001). These peptides recognize GW5638/GW7604-ERα complexes but not tamoxifen-ERα or other ligand bound ER complexes (Willson et al., 1994; Willson et al., 1997), indicating that conformational changes elicited by GW5638 and tamoxifen are different. To better understand the pharmacology of GW5638, we solved the crystal structure of ERα ligand binding domain (LBD) bound to GW5638. The crystal structure reveals a new LBD conformation in which AF2 H12 is repositioned by direct contacts between the carboxyl side chain of GW5638 and the N terminus of H12. The associated decrease in ERα stability may account for the ability of GW5638 to inhibit tamoxifen-resistant MCF-7 breast tumor explants.

Results

GW5638 Induces a Distinct Conformation in H12 and L11-12 through Specific Interactions with H12

The structure of ERα LBD bound to GW5638 was solved by molecular replacement with a truncated raloxifene-ERα LBD structure to a resolution of 2.7 Å with an R factor of 0.208 and free R factor of 0.236 (Table 1). The refined structure contains three GW5638-ERα LBD complexes in one asymmetric unit and reveals clear electron density for GW5638 (Figure 1C, left). Like other known ER LBD structures, GW-ERα LBD folds into a canonical three-layered sandwich of twelve α helices. GW5638 lies in an orientation similar to OHT inside the ligand binding pocket (Figures 2A and 2B). Surprisingly, the carboxyl groups between GW5638 and Asp 351

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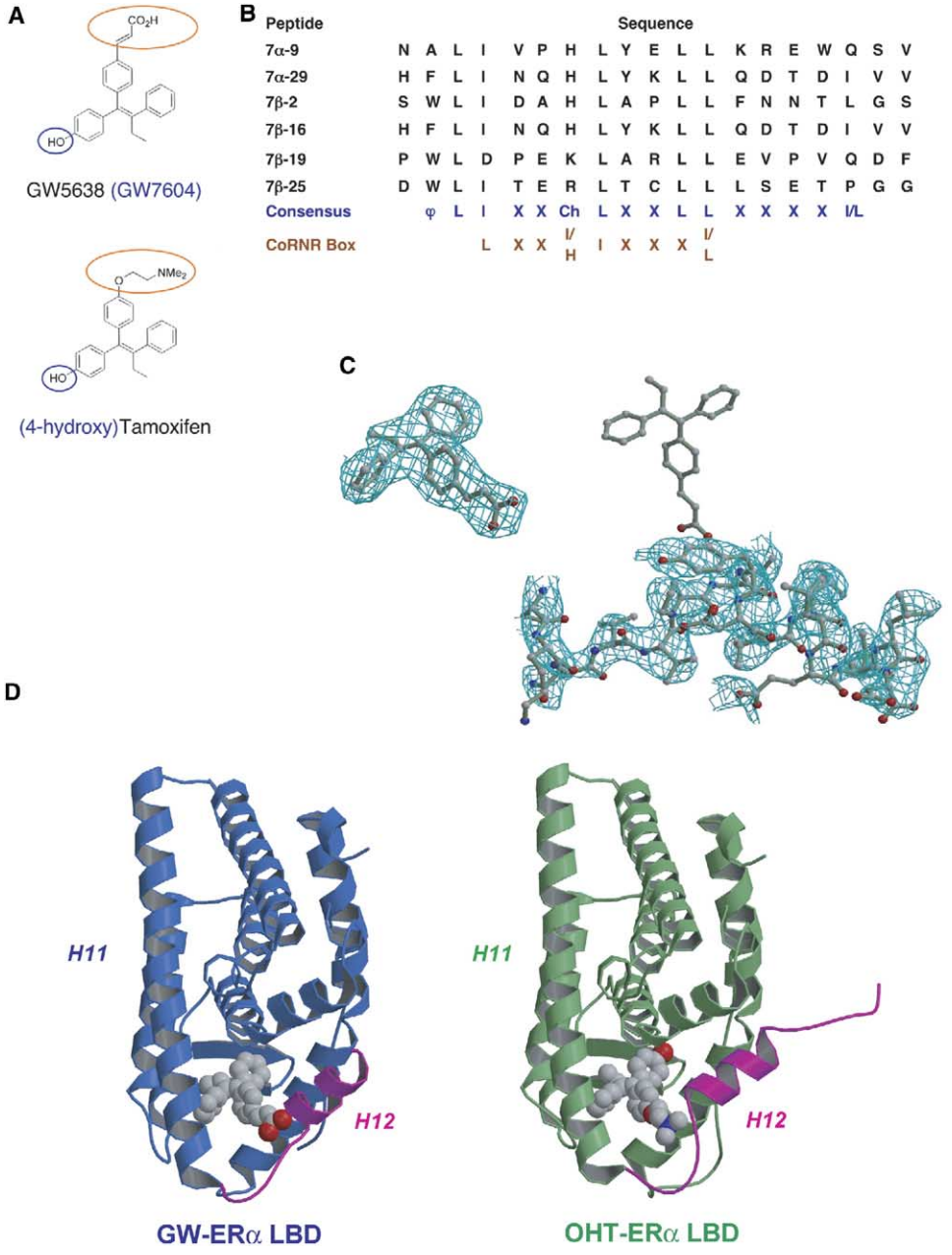

Figure 1. Overall Structure of GW5638-ERα LBD Compared to OHT-ERα LBD

(A) Structures of tamoxifen and GW5638 (OHT and GW7604 with a hydroxyl group at the fourth position). The side chains of both compounds are highlighted in orange.

(B) Sequence alignment of the GW-selective peptides and CoRNR box motif. Abbreviations and symbols used are as follows: ϕ, hydrophobic residue; Ch, charged residue; X, any amino acid.

(C) Two Fo-Fc electron density maps of GW5638, H12, and L11-12 calculated at the resolution of 2.7 Å and contoured at 1.0 σ.

(D) Equivalent views of GW-ERα LBD (left) and OHT-ERα LBD (right). H12 in the two structures is colored magenta.

form a hydrogen bond at the crystallization pH of 5.6 (Figure 2A), rather than repelling each other as predicted from molecular modeling studies of the 4-hydroxy metabolite GW7604 (Bentrem et al., 2001). It is likely that the hydrophobic environment of the pocket results in protonation of the weakly acidic acrylate, allowing for hydrogen bond formation with Asp351 (Urry et al., 1992). Interestingly, the acrylate side chain of GW5638 induces an unexpected conformation in H12, the C-terminal "activation function-2" (AF2) helix, which has not been observed in other antagonist bound NR LBD structures (Figure 1D). The electron density in this region was clearly revealed in the initial omit map and the final 2Fo-Fc map is shown at the right section of Figure 1C. In the OHT and RAL ERα LBD structures, H12 binds to and occludes the coactivator binding site by mimick-

Table 1. Statistics for GW5638-ERα LBD Structure Determination

Data Collection Details	
Wavelength	λ = 0.9
Space group	P6(1)22
Unit cell dimension	a = b = 136.031 Å; c = 357.626 Å
Processing Statistics	
Resolution range	100-2.70 Å
Observations	408320
Unique reflections[a]	53588 (5227)
Completeness[a]	97.3% (96.8%)
I/σ (last shell)	26.2 (2.1)
R_{merge}[b]	7.2%
Refinement Statistics	
Resolution range (Å)	27.95-2.70 (2.77-2.70)
Reflections used (R_{free} set)	45583 (5160)
Total nonhydrogen atoms	5756
R_{cryst}(%)[a,c]	20.8 (34.8)
R_{free}(%)[a,c]	23.6 (35.5)
Rmsd deviation	
Bonds (Å)	0.014
Angles (°)	1.34
Average B factor (Å^2)	45.9

[a] The value in parentheses corresponds to the highest resolution shell (2.77–2.70 Å).
[b] $R_{merge} = 100 \times \Sigma|I - \langle I \rangle| / \Sigma\langle I \rangle$.
[c] $R_{cryst} = \Sigma|F_{obs} - F_{calc}| / \Sigma|F_{obs}|$, where F_{obs} and F_{calc} are observed and calculated amplitudes, respectively. R_{free} is calculated similarly using a test set (10.2%) of reflections.

ing the hydrophobic interactions of coactivator NR box LXXLL motifs with the recognition groove formed by H3, H4 and H5 (Brzozowski et al., 1997; Shiau et al., 1998). This orientation of H12 is partially displaced from the hydrophobic cleft in the GW-ER complex, which can be attributed to the water-mediated hydrogen bonds that link one of the carboxyl oxygen atoms in GW5638 to the backbone NH groups at the N terminus of H12 (Figure 2C).

GW5638 Induces a Relocation of Hydrophobic Residues in H12

Leu-536 mediates the N-terminal capping of H12, which is initiated by the water-mediated hydrogen bonds between the GW 5638 acrylate carboxyl group, Leu-536 and Tyr-537, bringing the N terminus of H12 and the loop connecting H11 and H12 (L11-12) 6–7 Å closer to the ligand (Figure 2C). Consequently, H12 relocates ~10° away from H11, compared to the OHT and raloxifene complexes, resulting in a ~50° difference in orientation for H12 in the GW-ERα and OHT-ERα structures (Figure 3A). This displacement disrupts the hydrophobic interactions between the AF-2 cleft and the H12 LLEML residues in the OHT-ER structure (Figure 4A). In addition, a series of hydrogen bonds between H3/H5 and H12 in the OHT-ER structure are lost in the GW-ER structure (Figure 4B). Significantly, this unusual orientation induces a repositioning of hydrophobic residues in H12 (Leu-536, Leu-539, Leu-540, and Met-543) such that the side chains of these residues are no longer buried in the hydrophobic core but become relocated to the protein exterior (Figure 3B). Thus, GW5638 causes a nearly 27% increase in the exposed hydrophobic surface of H12 compared to the OHT-ERα structure. This surface is stabilized in part by contact with a neighboring LBD molecule in the crystal lattice.

Analysis of the Surface Hydrophobicity of ERα LBD

A bis-ANS probe that fluoresces strongly in hydrophobic environments (Rosen and Weber, 1969; Slavik, 1982) was used to determine if structure-predicted surface hydrophobicity is reflected in the solution conformations of ERα LBD in the presence of different ligands. Based on peptide phage-display and mammalian two-hybrid data, GW7604 and GW5638 should induce nearly identical conformations in the ERα LBD (Connor et al., 2001). Because GW7604 has a significantly higher affinity for ERα than GW5638 (Willson et al., 1997) and is more like the OHT structure, we used GW7604, OHT, ICI 182,780, and the natural ERα agonist 17β-estradiol (E2) in bis-ANS experiments. The binding of bis-ANS to ERα LBD was monitored by the enhancement of bis-ANS fluorescence intensities (Rosen and Weber, 1969; Slavik, 1982). Consistent with the structure-based prediction (see Figure 5A), the fluorescence intensity for GW7604-ERα LBD was ~20% greater than for OHT-ERα LBD. The ligand-mediated steady-state surface hydrophobicity of ERα LBD in solution was ICI > E2 > GW7604 > OHT. (Figure 5B). For ICI-ERβ LBD (Brzozowski et al., 1997; Pike et al., 2001), the bulky ICI side chain blocks the association between H12 and the rest of the LBD (Figure 5A). Thus, ICI-ER LBD should have the most open hydrophobic interior. E2-ER LBD has an agonist bound conformation (Brzozowski et al., 1997) that also exposes a hydrophobic binding site for coactivator NR box domains (Shiau et al., 1998).

Instability of ERα Is Associated with Surface Hydrophobicity

Several studies have shown that ERα protein degradation is mediated through the ubiquitin-proteasome pathway. Agonist-induced ERα protein turnover is concomitant with transcriptional activation and coactivator recruitment (Dace et al., 2000; Nawaz et al., 1999a; Shao et al., 2004), whereas degradation induced by the potent antagonist ICI 164,380/182,780 presumably occurs through a different mechanism and OHT appears to stabilize ERα. Surface hydrophobicity has long been associated with other destabilizing modifications such as oxidation, arginylation, and ubiquitination, and this property is recognized as an important determinant of protein stability (Bohley, 1996). Our data suggest that ERα LBD surface hydrophobicity elicited by various antagonists (Figure 5B) may correlate with ERα stability. We therefore inspected the protein level of ERα by Western blot analysis after 4 hr of ligand treatment. As reported elsewhere, GW7604 and ICI 182,780 differentially reduce ERα steady-state levels, whereas OHT stabilizes ERα, despite the structural similarity between GW5638/7604 and tamoxifen (Figure 5C). Our data show an inverse correlation between the surface hydrophobicity and ERα protein levels after short-term ligand treatment (Figure 5D). ICI 182,780, which has the most profound effect on ERα degradation, induces a conformation that exposes the most surface hydrophobicity

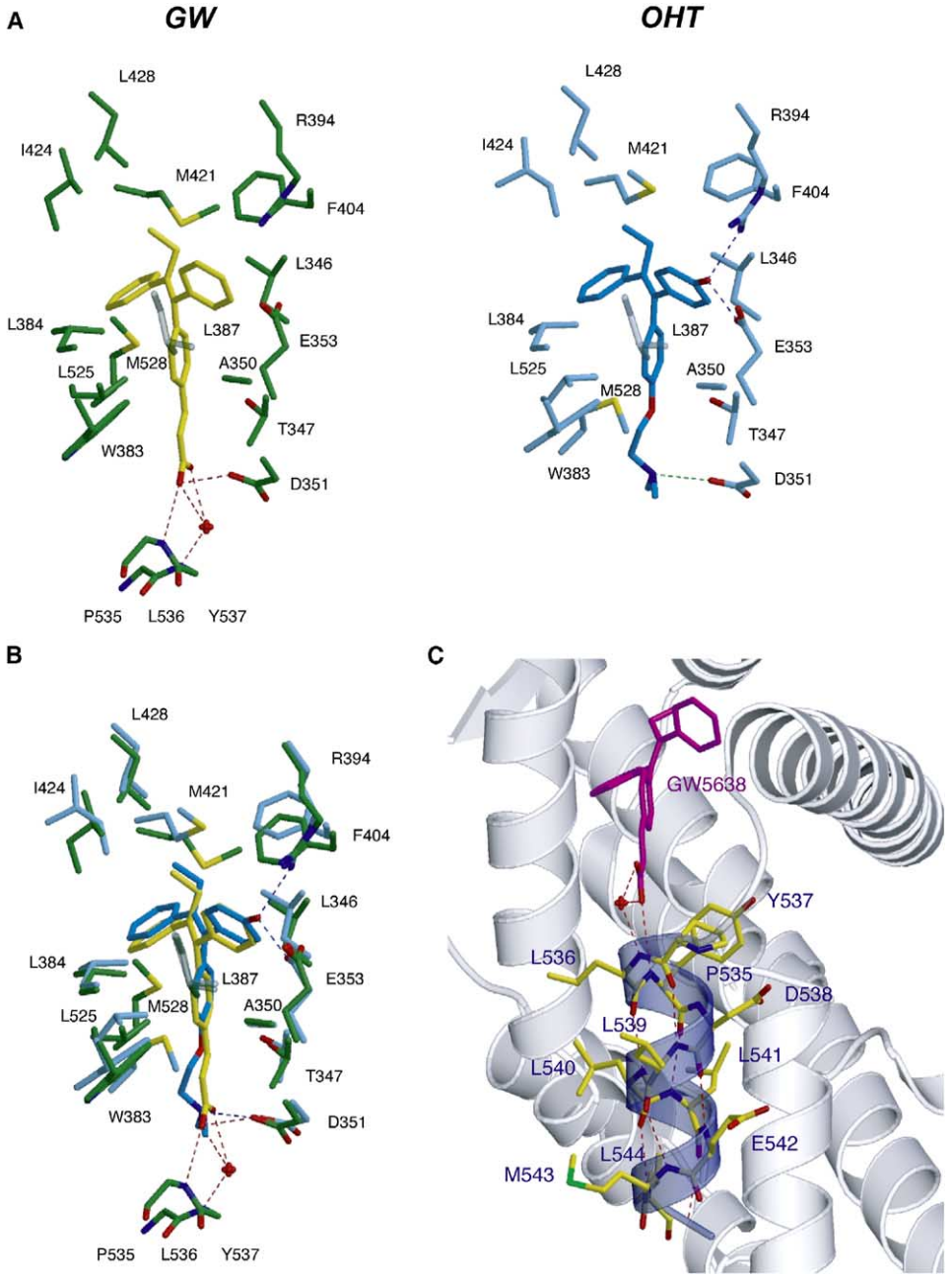

Figure 2. Ligand-LBD Interactions in GW5638-ERα LBD and OHT-ERα LBD

(A) Interactions of GW5638 or OHT and the ligand binding pocket of ERα LBD (4.0 Å cutoff). GW5638 is colored yellow, and OHT is colored blue. Atoms of resides are colored by atom type (carbon [GW], green; carbon [OHT], light blue; nitrogen, dark blue; oxygen, red; sulfur, yellow). Side chains of all residues are shown except that main chains of residues P535, L536, and Y537 in GW-ER are illustrated. For clarity, the side chain of M343 is not shown. Hydrogen bonds are depicted as dashed red lines in GW-ER and dashed blue lines in OHT-ER. The weak salt bridge between the dimethylamino group of the OHT side chain and the carboxylate side chain of D351 is colored green.

(B) An overlay of GW5638-ERα LBD and OHT-ERα LBD in the ligand binding pocket.

(C) The side chain of GW5638 initiates N-terminal capping of H12. Hydrogen bonds are depicted as dashed red lines. GW5638 is colored magenta. Residues in H12 are colored by atom type (carbon, yellow; nitrogen, dark blue; oxygen, red; sulfur, green).

compared to other ligands (Figures 5B and 5D). OHT, which decreases ERα turnover, has the least exposed hydrophobic surface, whereas GW7604 induces a conformation that is intermediate in exposed hydrophobic surface, consistent with its effect on ERα stability (Figure 5D). Although E2 appears to induce an exposed hydrophobic surface that correlates with its effect on ERα stability, this surface is presumably occupied by coactivator in vivo, which appears to be essential for agonist-mediated degradation of ERα. Overall, these results

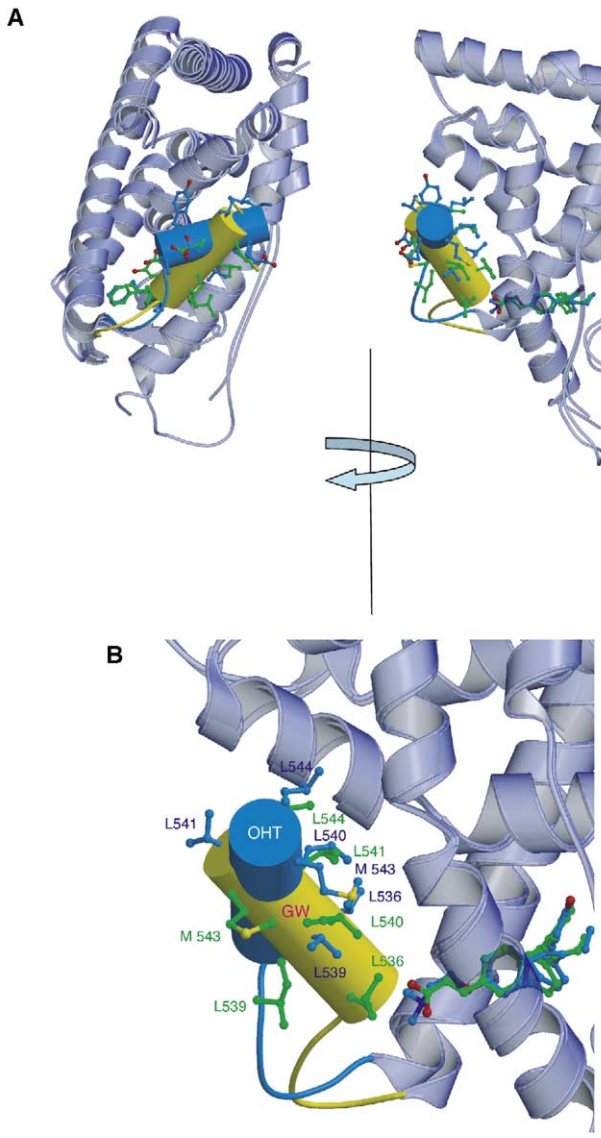

Figure 3. Superimposed Structures of GW5638-ERα LBD and OHT-ERα LBD

(A) Two orthogonal views of the orientations and positions of H12 and L11-12. H12 is shown as cylinder presentation, yellow in the GW complex and blue in the OHT complex. Side chains in H12 are colored by atom type (carbon [GW], green; carbon [OHT], blue; nitrogen, dark blue; oxygen, red; sulfur, yellow).

(B) A closer look of the superimposed GW and OHT-ERα LBD structures. Only hydrophobic residues in H12 are shown. Side chains and ligands are colored by atom type (carbon [GW], green; carbon [OHT], blue; nitrogen, dark blue; oxygen, red; sulfur, yellow).

suggest that antagonist-mediated increases in surface hydrophobicity can contribute to ERα instability. Consistent with this hypothesis, replacement of Leu-536, Leu-539, and Leu-540 with Gln, which should reduce the surface hydrophobicity of H12, increased ERα stability from 68% to 85% in the presence of GW7604 (Figure 5E).

Mutations in the Putative CoRNR Box-Interacting Region of ERα Affect the Binding of GW-Specific Peptides

GW-selective peptides, which were obtained from a phage library that contained the "LXXLL" motif, share homology with the NR box-related consensus corepressor nuclear receptor-interacting motif (CoRNR box: LXXI/HIXXXI/L) (Perissi et al., 1999) (Figure 1B). To test if these peptides bind to the putative CoRNR binding region in the ERα LBD, which partially overlaps with the coactivator binding site in the AF-2 cleft, we used a mammalian two-hybrid approach. Peptides were fused to the DNA binding domain of Gal4 and the ERα LBD was fused to the VP16 activation domain. Several mutations that are known to enhance or reduce the interaction of thyroid hormone receptor (TR) or retinoic X receptor (RXR) with NCoR/SMRT (Hu and Lazar, 1999) were introduced into ERα at equivalent positions (Figure 6A). ERα-peptide interaction was determined by the ability of the wild-type or mutant ERα-VP16 to activate transcription from the Gal4-responsive reporter in the presence of the GW compound. Deletion of H12 (ERα 537X or ERα 538X), which was previously shown to increase NCoR binding to ERα (Webb et al., 2003), enhanced the binding of GW-selective peptides to GW-ERα LBD (Figure 6B). Although the L372R and V376R mutations did not significantly affect peptide binding (data not shown), mutations L379R, at the base of H5, and K362A, at the H3/H4 boundary, significantly reduced these interactions (Figures 6C and 6D). L379R is

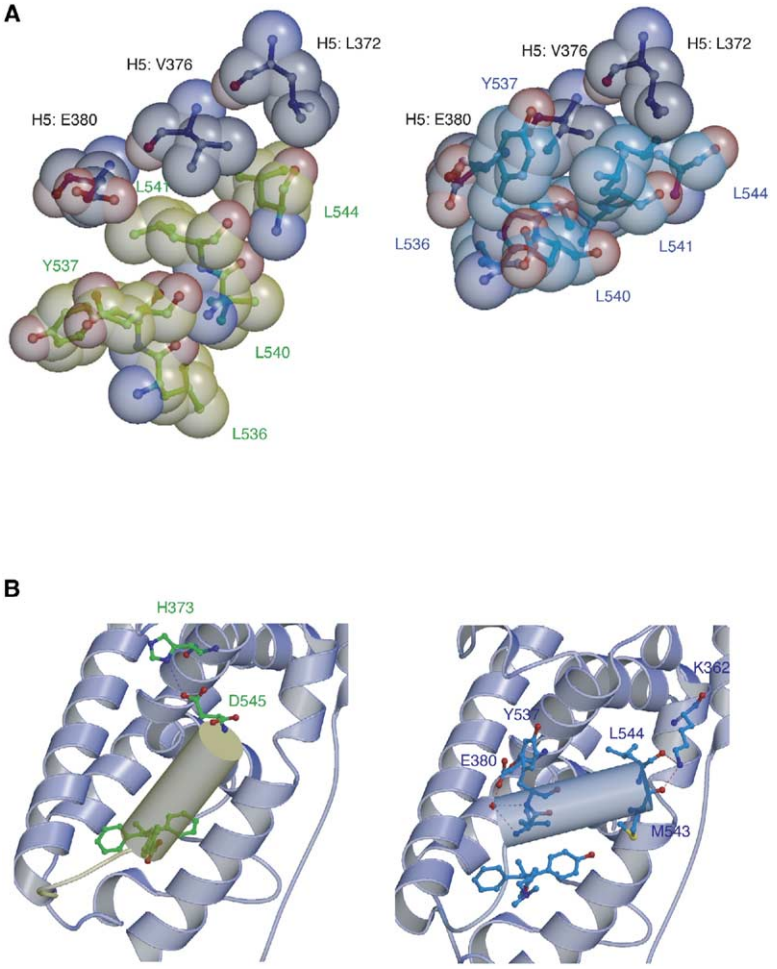

Figure 4. Interactions between H12 and H3/H5 in GW5638-ERα LBD and OHT-ERα LBD

(A) van der Waals contacts between H5 and H12 in the GW-ER (left) and OHT-ER (right). Residues are colored by atom type (carbon [H5], dark blue; carbon [H12 in GW], green; carbon [H12 in OHT], light blue; nitrogen, blue; oxygen, red).

(B) Illustrations of GW and OHT-ERα LBD showing the interactions between H3/H5 and H12. The hydrogen bonds in the OHT-ER structure are depicted as dashed red lines. The weak salt bridge in the GW-ER structure is depicted as blue dashed line. Side chains and ligands are colored by atom type (carbon [GW], green; carbon [OHT], blue; nitrogen, dark blue; oxygen, red; sulfur, yellow).

of interest because this residue was shown to be critical for NCoR recruitment to ERα (Webb et al., 2003). In the GW-ERα LBD crystal structure, H12 is partially displaced from the AF2 cleft, which may facilitate corepressor competition with H12 in the presence of GW5638/7604 compared to tamoxifen (Figures 3 and 4). The LBD structures correlate well with fluorescence-microsphere binding data (Iannone et al., 2004), which showed that GW7604 and other compounds with the same acrylate or carboxylate side chain are more effective recruiters of corepressor-like peptides to ERα. Although we did not detect a significant interaction between SMRT/NCoR and ERα in the mammalian two-hybrid assay (data not shown), it is possible that the GW-selective peptides are affinity-optimized versions of the CoRNR box peptide motif for GW-ERα.

Discussion

In total, the GW5638-ERα LBD structure and supporting data may explain how GW5638/7604 is an effective inhibitor of tamoxifen-resistant MCF-7 tumor explants. Clearly, relatively subtle ligand modifications can significantly alter the conformation of the H12 molecular switch. In addition to preventing coactivator recruitment by occlusion of the AF2 cleft, similar to OHT and RAL, GW5638/7604 also destabilizes ERα, although less so than the more potent ER antagonist ICI 164,380/182,780. This effect is associated with a rotation of H12, induced by the tethering of Leu-536 and Tyr-537 to the carboxyl moiety on GW5638/7604, which leads to an increase in the surface hydrophobicity of the ERα LBD. This increased surface hydrophobicity is associated with a decrease in ERα stability. Therefore, GW5638/7604 is a more potent growth inhibitor than OHT and RAL because it also downregulates ERα.

The Conformation of H12 Plays a Role in the Stability of ERα

The acrylate side chain of GW5638/7604, a tamoxifen analog, interacts with Leu-536 and Tyr-537 of the ERα LBD to induce capping of H12 and an unexpected conformation for this AF2 molecular switch. The resulting increase in ERα surface hydrophobicity correlates with a decrease in receptor stability, in sharp contrast to the increased stability observed for OHT-ERα. Notably, the ERα was significantly stabilized in the presence of GW7604 (Figure 5E) by replacement of Leu-536, Leu-539, and Leu-540 with Gln, which should reduce the surface hydrophobicity of H12. Thus, the GW5638-ERα

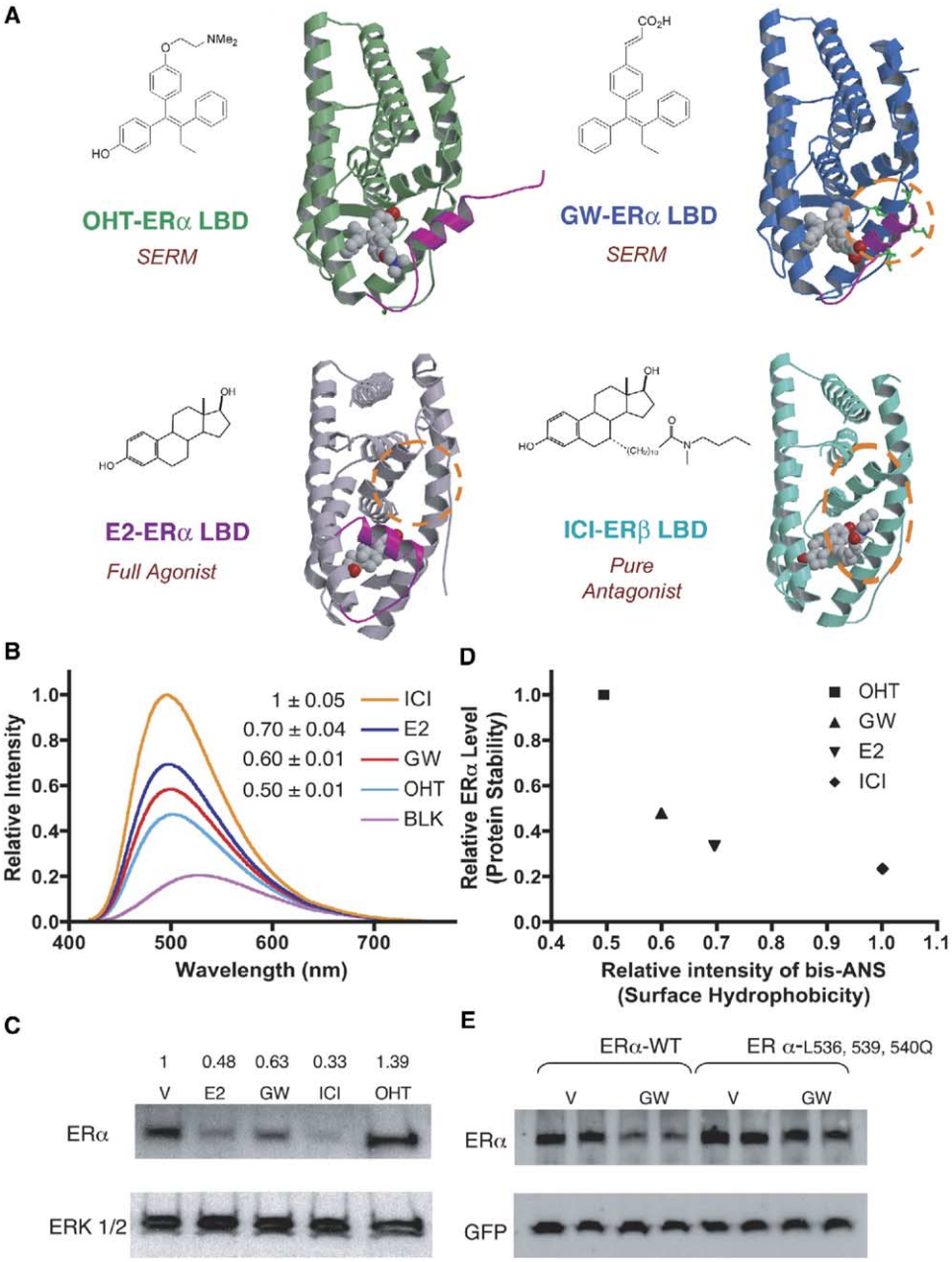

Figure 5. Surface Hydrophobicity Contributes to the Stability of Antagonist Bound ERα

(A) Overall structures of E2- (Brzozowski et al., 1997), OHT- (Shiau et al., 1998), GW-ERα LBD, and ICI-ERβ LBD (Pike et al., 2001). The regions that contribute to the altered surface hydrophobicity are circled in orange. H12 and L11-12 in the structures are colored magenta.

(B) The fluorescence intensity of bis-ANS in the presence of buffer alone (BLK), ERα LBD bound to E2, OHT, GW7604, or ICI. The relative fluorescence intensities of triplicate samples after normalized to BLK are expressed in numerical values as mean ± standard deviation compared to ICI.

(C) Protein levels of ERα in MCF-7 cells after 4 hr of ligand induction. The Western blot signals of ERα were normalized to the internal control ERK1/2 protein expression and the relative quantification is shown in numerical values (V, ethanol; E2, 17β-estradiol; GW, GW7604; ICI, ICI 182,780; OHT, 4-hydroxytamoxifen).

(D) Correlation between the surface hydrophobicity and the protein level of ERα after short-term ligand treatment.

(E) The Western blot showing the expression levels of the wild-type or mutant ERα. Ishikawa cells were cotransfected with the wild-type or mutant ERα and a control green fluorescence protein expression vector (V, ethanol; GW, GW7604).

structure adds another level of complexity to the observed conformational flexibility of H12 by showing that it not only controls the recruitment of cofactors but also influences the stability of ERα. Overall, our data suggest that antagonist-mediated increases in surface hydrophobicity can contribute to ERα instability.

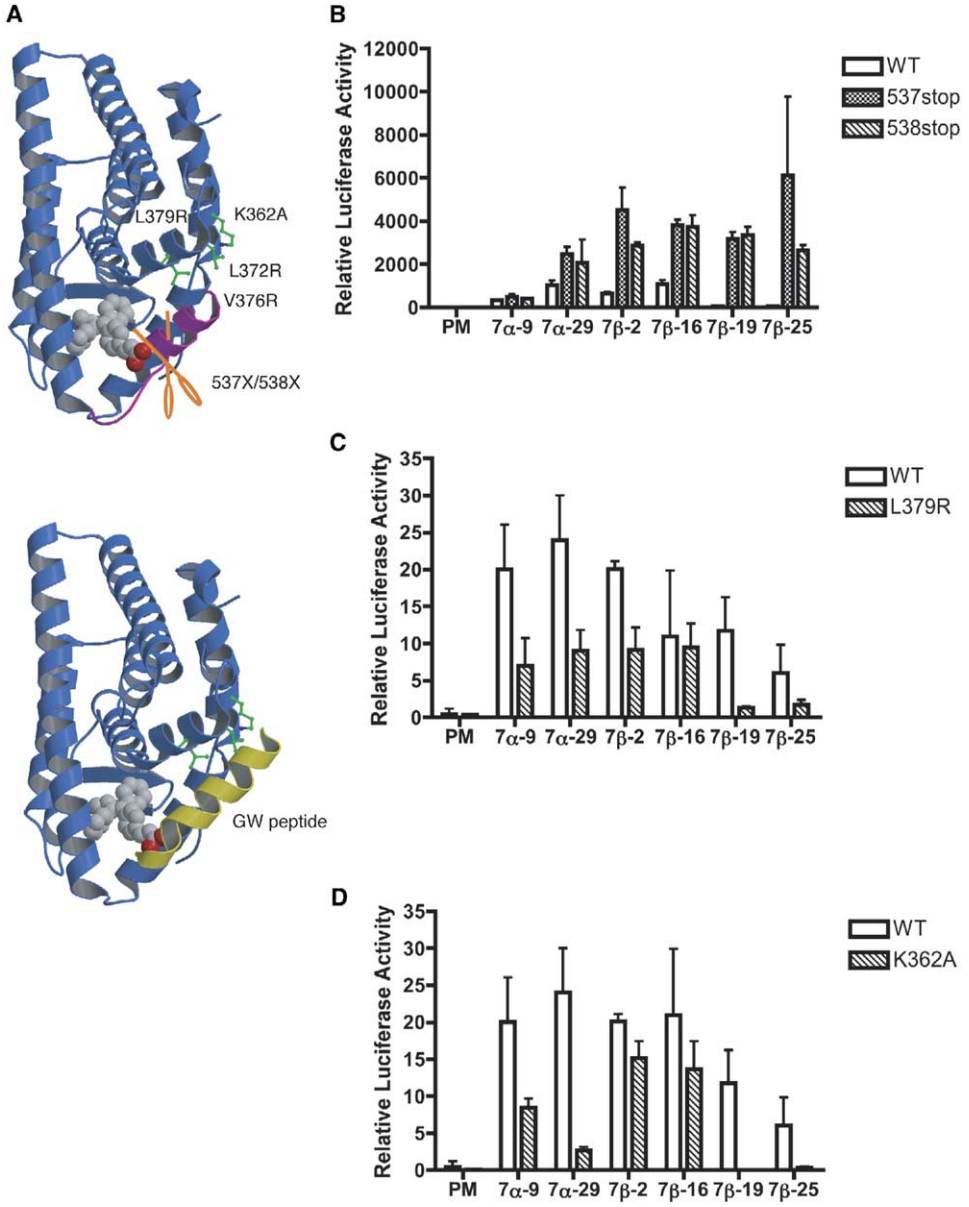

Figure 6. Mammalian Two-Hybrid Data Showing How Mutations in the Putative CoRNR Box-Interacting Region of ERα Affect the Binding of GW-Specific Peptides

(A) The upper section shows mutations introduced in this study; the lower section shows GW 7β-16 peptide binding to GW-ERα LBD. H12 is omitted for clarity. The model of GW 7β-16 peptide was generated using SwissPDB viewer (Guex and Peitsch, 1997) by replacing the sequence of a canonical α-helix to GW 7β-16. The position of GW 7β-16 peptide is superimposed on H12 in the OHT-ER structure.

(B) H12 ERα truncations increase the binding of GW-selective peptides to GW-ERα. COS-7 cells were transfected with VP16-ERα, VP16-ERα 537X or VP16-ERα 538X, Gal4-DBD-peptide fusion, and 5xGal4-TATA-Luc. The error bars indicate the standard deviation of triplicate samples.

(C) ERα mutant L379R reduces the binding of GW-selective peptides to GW-ERα. COS-7 cells were transfected with VP16-ERα, VP16-ERα L379R, Gal4-DBD-peptide fusion, and 5xGal4-TATA-Luc. The error bars indicate the standard deviation of triplicate samples.

(D) The ERα mutant K362A reduces the binding of GW-selective peptides to GW-ERα. COS-7 cells were transfected with VP16-ERα, VP16-ERα K362A, Gal4-DBD-peptide fusion, and 5xGal4-TATA-Luc reporter. The error bars indicate the standard deviation of triplicate samples.

The exposed hydrophobic surface of H12 is partly stabilized via crystal contacts with an adjacent GW5638-ERα LBD monomer. Other published ERα LBD structures, such as the OHT-ER LBD complex (Shiau et al., 1998), also show similar packing arrangements. In the absence of a solution structure, it is difficult to determine the net contribution of such contacts to the observed conformation. However, the bis-ANS data (Figure 5B) support the conclusion that GW-ER has a larger exposed hydrophobic surface than OHT-ER.

It is also unlikely that the acidic pH (5.6–5.7) at which GW5638-ERα LBD crystallized contributed significantly to the observed structure. OHT-ERα LBD (Shiau et al., 1998) and RAL-ERα LBD (Brzozowski et al., 1997) have

very similar structures, even though the former crystallized under acidic conditions (pH 6.5) and the latter crystallized at pH 8.5. The biochemical and cell-based experiments (Figures 5 and 6), which were carried out at pH 7–8, are consistent with the behavior predicted by the GW5638-, OHT-, E2-, and ICI-ERα LBD structures (Figure 5A).

Estradiol-mediated ERα degradation has been linked to coactivator recruitment and transcriptional activation, which is not involved in GW- or ICI-mediated ERα degradation and inactivation (Lonard et al., 2000; Lonard and Smith, 2002; Nawaz et al., 1999a; Reid et al., 2003; Shao et al., 2004; Wijayaratne and McDonnell, 2001). The mechanisms for agonist- and antagonist-regulated ERα degradation appear to be distinct (Shao et al. 2004), although ubiquitin-mediated targeting of ER to the 26S proteasome is undoubtedly essential for both, and receptor stability and the level of ERα ubiquitination have been linked (Wijayaratne and McDonnell, 2001).

Hydrophobic clusters are considered to be important recognition motifs for ubiquitin E3 ligases, (Bohley, 1996). One of the unexpected findings in this study was the correlation between ERα LBD surface hydrophobicity in solution and subtle alterations in the conformation of H12 induced by different ligands. Surface hydrophobicity is not only important for E3 ligase recognition and ubiquitination but also for the targeting of multiubiquitinated proteins to the 26S proteasome (Bohley, 1996). We propose that the degree of LBD surface hydrophobicity observed for different ligands may correlate with differential levels of ERα ubiquitination. Ubiquitin can also function as a "chaotropic" signal by unfolding target molecules and exposing more hydrophobic residues, thereby amplifying the degradation process. Several components of the ubiquitin-proteasome system, including E3 ligases, are reported to be involved in the degradation of steroid receptors, such as uba3 (Fan et al., 2002), Ubc9 (Poukka et al., 1999), RSP5/RPF1 (Imhof and McDonnell, 1996), MDM2 (Saji et al., 2001), SUG1/Trip1 (Lee et al., 1995; vom Baur et al., 1996), and E6-AP (Nawaz et al., 1999b). It is unlikely that GW- and ICI-mediated ERα degradation involves the same E3 ligase(s) as estradiol, although it is possible that the same E3 ligase targets GW- and ICI-ERα. However, since the conformations and transcriptional responses triggered by these two ligands are not the same in different tissue contexts, it is reasonable that different E3 ligases are involved. Experiments are ongoing to identify the ligases and other components that participate in the GW- and ICI-mediated ERα degradation.

Corepressor Recruitment and Antiestrogen Action

Transcriptional regulation by ERα is a complex process that involves the participation of coactivators and corepressors. While the interaction of coactivators with ERα is well established, the interaction and importance of corepressors is less clear (Dobrzycka et al., 2003). ERα is different from other NRs, such as retinoic acid receptor (RAR) and thyroid hormone receptor (TR) because it does not appear to actively repress transcription in the absence of ligand (Chen and Evans, 1995; Dobrzycka et al., 2003). However, evidence suggests that antagonist-mediated inhibition of ERα not only blocks coactivator recruitment but also facilitates the recruitment of corepressors to an actively repressed ERα complex (Cottone et al., 2001; Dobrzycka et al., 2003; Shang and Brown, 2002; Webb et al., 2003).

In the absence of an ERα-corepressor structure, our structural data suggest the possibility that corepressor-like peptides could compete with the partially displaced H12 for the AF-2 cleft in the GW-ERα structure. Our mammalian two-hybrid data (Figure 6) further support the structural information, in that deletion of H12 as well as two ERα mutations L379R and K362A significantly alter the binding of GW-specific peptides.

The mammalian two-hybrid data showed that the GW compound is more effective in recruiting corepressor-like peptides, suggesting that the distinctively oriented H12 might be more flexible in GW-ER. We do not rule out the possibility that the unusual H12 orientation in the observed GW-ER structure is a transitory instead of the equilibrium structure. While this structure reflects the accessible ER conformation in solution, a larger hydrophobic surface may imply the instability of H12 conformation upon GW binding. This may also explain why the GW compound is a more successful drug in recruiting corepressor-like peptides than OHT. Notably, these peptides were obtained in the absence of any structural information. The fact that some of these peptides are homologous to the CoRNR box motif suggests that the ERα conformation induced by GW5638 favors the recruitment of corepressor-like proteins, implying that corepressors could play a critical role in the pharmacology of GW5638.

GW5638 As a Prototype Drug for Tamoxifen-Resistant Breast Cancers

The mixed agonist/antagonist properties of SERMs and relatively mild side effects explain why drugs like tamoxifen and raloxifene are used for the treatment or prevention of breast cancer and for the prevention of osteoporosis, respectively. Tamoxifen is the standard endocrine treatment for ERα-positive primary and metastatic breast cancers. Unfortunately, most of these cancers become resistant within 2–5 years, and the risk of uterine cancer increases in women who take tamoxifen. Available pharmacological data show similar activities for raloxifene, but data on the activity of raloxifene in patients with advanced disease are limited (Buzdar et al., 1988; Gottardis and Jordan, 1987; Poulin et al., 1989). The commonly used second-line endocrine therapy for tamoxifen-refractory tumors includes aromatase inhibitors, gonadotrophin releasing hormone agonists, or the pure ERα antagonist ICI (Fulvestrant, Faslodex). However, these agents do not have the beneficial agonist activities associated with tamoxifen.

GW5638/7604 belongs to a class of molecules that has mixed function (SERM/SERD) (McDonnell, 2005). SERMs function either as agonists or antagonists, depending on the coregulator context. SERDs (selective estrogen receptor downregulators), like ICI and ZK-703 (Hoffmann et al., 2004), act as more potent antagonists by inducing receptor turnover. ICI is the only FDA-approved SERD for treating postmenopausal women with ER-positive metastatic breast cancers that no

longer respond to tamoxifen. Interestingly, GW5638/7604 shares some similarities with ICI besides its effect on ER stability. ICI and GW5638/7604 derive from preexisting ER ligands (estradiol and tamoxifen). They both induce different ER conformations than their parent ligands, through side chain modifications. In addition, the destabilization of ER induced by ICI and GW5638/7604 is brought about, at least in part, by an increase in the surface hydrophobicity of ER.

GW5638/7604 shares some of the HRT benefits of tamoxifen but acts as a more potent antagonist in the breast and does not stimulate the uterus. The data presented here show that the distinct pharmacologies of tamoxifen and GW5638 are due, at least in part, to subtle changes in the respective ERα AF2 conformations. These data also suggest that an acidic side chain may be a useful substitute for drug design on the triphenylethylene scaffold. If proven to be effective in clinical trials, molecules like GW5638/7604 that could be used as a second-line therapy for patients whose breast cancers have become tamoxifen resistant. Because no single endocrine agent is likely to prevent recurrent disease in ER-positive breast tumors, there continues to be a need for novel agents that differentially target estrogen/ER-signaling pathways.

Experimental Procedures

Chemicals, Materials, and Plasmids

GW5638 and GW7604 were synthesized at GlaxoSmithKline (Research Triangle Park, North Carolina) by using the published procedure (Willson et al., 1994). 17β-Estradiol and 4-hydroxytamoxifen were obtained from Sigma-Aldrich (St. Louis, Missouri). ICI 182,780 was purchased from Tocris (Ellisville, Missouri). Bis-ANS was from Molecular Probes, Inc. (Eugene, Oregon). The generation of plasmids including the wild-type VP16-ERα, mutants VP16-ERα (K362A, L372R, and V376R), as well as Gal4-DBD-peptide fusion was described previously (Huang et al., 2002). Mutant receptor VP16-ERα L379R as well as the triple mutant receptor VP16-ERα L536, 539, 540Q were generated using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, California) with the wild-type VP16-ERα as template. VP16-ERα 537X and VP16-ERα 538X were constructed by PCR using the wild-type ERα as template with primers containing a stop codon at ERα 537 or ERα 538 and were subsequently subcloned into the EcoRI sites of the pVP16 vector.

Protein Expression and Purification

The human ERα LBD (residues 297–554) was expressed in BL21(DE3)pLysS as described previously (Shiau et al., 1998). The amount of ERα LBD in each bacterial extract was determined by saturation analysis with ^{3}H-estradiol using a controlled pore glass-bead (CPG) assay. Protein purification was carried out on an estradiol-Sepharose affinity column (E-Seph) (Greene et al., 1980), followed by FPLC ion exchange purification (Resource Q, Pharmacia, Sweden). While still bound to the E-Seph beads, ERα LBD was caboxymethylated with 5 mM iodoacetic acid (Hegy et al., 1996). GW5638-containing buffer was used to elute ERα LBD from the affinity column. Protein samples were analyzed by native gel electrophoresis. FPLC fractions containing the purest protein were collected and concentrated to 5–10 mg/ml for crystallization.

Crystallization and Data Collection

The crystals of GW5638-ERα LBD were grown at 4°C by hanging drop vapor diffusion. Samples (2 μl) of 5 mg/ml protein were mixed with 2 μl of the reservoir buffer consisting of 1.5%–2% ethylene imine polymer, 100 mM trisodium citrate (pH 5.6–5.7), 0.5 M sodium chloride, and 9% of Yttrium chloride hexahydrate. Crystals that grew up to the size of >0.1 mm were harvested and transferred to a cryoprotectant solution containing 2% ethylene imine polymer, 100 mM trisodium citrate (pH 5.6), 1M of sodium chloride, and 25%–40% glycerol and stored in liquid nitrogen. Data were collected at BioCARS 14BMC beamline (λ = 0.9 Å) station, Advanced Photon Source, Argonne National Laboratory. Diffraction data were recorded on an ADSC Quantum-4 detector. The images of data sets were processed with Denzo and Scalepack programs in the HKL 1.96 package.

Structure Determination and Refinement

Our initial efforts to determine the structure utilized a low-resolution (3.3 Å) data set. The three ligand binding domains in the asymmetric unit were located by molecular replacement with EPMR version 2.5 (Kissinger et al., 1999) using a modified raloxifene-ERα LBD (1ERR.pdb) as the search probe. The crystals of the GW5638-ERα LBD lie in the space group of $P6_122$. The unit cell parameters are a = b = 136.031 Å and c = 357.626 Å. The R factor and correlation coefficient (CC) after rigid body refinement are 43.1% and 0.65, respectively. The model was refined initially with CNS 1.0 (Brunger et al., 1998) and later with Refmac5.1.24 (Murshudov et al., 1997). TLS (Schomaker and Trueblood, 1968) in Refmac was applied to correct for anisotropic displacements of each monomer in the asymmetric unit. Model building of the GW5638-ERα LBD structure that was not included in the original search probe from molecular replacement was done by Xtalview (McRee, 1999). The R_{free} set contains a random sample of 10.2% of all data. The structure of GW5638-ERα LBD using data up to 2.7 Å was refined to a crystallographic R factor of 20.8% and an R_{free} factor of 23.6%. The statistics of the structure and data sets are summarized in Table 1.

Structural Analysis and Illustrations

The angles between the aligned H12 from different complex structures (OHT-ER and GW-ER) were calculated using a python script developed in-house based on CCL (http://renzresearch.com). The angle between any two helices is defined as the spatial angle between the axes of the two helices. Illustrations of structures were generated using Molscript (Kraulis, 1991) or Bobscript (Esnouf, 1999), which were further rendered by Raster3d (Merritt, 1994).

bis-ANS Probing of Receptor Surface Hydrophobicity

All ligand bound ERα LBD samples were prepared as described above. Samples of ERα-LBD were purified on E-Seph to separate ligand bound from unliganded receptor. Eluted protein samples were dialyzed against 25 mM of Tris (pH 8.1) and 25mM of sodium chloride buffer overnight in order to remove DTT and excess ligand that could potentially interfere with the bis-ANS binding study. 1.2 μM of protein sample were incubated with 25 mM of bis-ANS at room temperature for 1 hr. An aliquot (200 μl) of the solution was transferred into a 3 × 3 mm quartz fluorescence cuvette and placed in the sample chamber of fluorolog Tau-2 Fluroescence Spectroscopy System (Spex industries, Inc., Edison, New Jersey). The samples were excited at 395 nm, and the emission spectra were scanned from 420–750 nm. The data were normalized to the blank and averaged from triplicate results. The peak intensity of each spectrum was normalized to the height of ICI-ERα LBD sample.

Cell Culture and Transient Transfections

COS-7 cells were cultured in DMEM (Mediatech, Inc., Herndon, Virginia) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, Georgia) and maintained in the 37°C incubator with 5% CO_2. The cells were plated into 48-well plates 24 hr before transfection. DNA was delivered to the cells by transient transfection using PolyFect. (Qiagen, Inc., Valencia, California). 100 nM of ligands were added to the cells 18–24 hr before the cell harvest. For mammalian two-hybrid assay, 200 ng 5xGal4-TATA-Luc reporter plasmid, 200 ng VP16-receptor fusion, 200 ng Gal4-DBD-peptide fusion, and 20 ng normalization plasmid pRL-TK or pCMV-βgal were used. 100 nM of GW7604 were added to the cells 18–24 hr before the cell harvest. Luciferase activities were normalized to either Renilla luciferase of β-galactosiase activities by Dual-Luciferase Reporter Assay or β-galactosidase Enzyme Assay System (Promega Corp., Madison, Wisconsin).

Western Blot Analyses

MCF-7 cells were maintained in phenol-free medium containing 10% charcoal-filtered serum at least 24 hr before the assay. 100 nM of ligands were added to the cells for 4 hr before harvest. Ishikawa cells were cultured in DMEM F12 HAM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Atlanta Biologicals). The cells were plated into 12-well plates 24 hr before transfection. DNA was delivered to the cells by transient transfection using Effectene according to the manufacturer's protocol. (Qiagen, Inc.). 10 μM of ligands were added to the cells 18 hr before the cell harvest. Proteins (20 μg samples) from the cell extracts were separated on SDS-PAGE and transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences Corp., Piscataway, New Jersey). The receptors were detected with the monoclonal antibody H222 (Greene et al., 1980). Total ERK1/2 were detected with p44/42 MAP kinase antibody (Cell Signaling Technology, Beverly, Massachusetts). The GFP expression was detected with polyclonal GFP (FL) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, California). Immunoreactive bands were visualized by enhanced chemiluminescence using Western Lightning Chemiluminescence reagent (PerkinElmer, Wellesley, Massachusetts) as described by the manufacturer.

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Accession Numbers

Coordinates of the GW5638-ERα LBD structure were deposited in the Protein Data Bank under the ID code 1R5K.

APPENDIX 2. Keystone Nuclear Receptor Symposia poster abstract

Structural Characterization of hERα-LBD in Complex with GW5638

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Breast cancers affect one in eight women in the United States. Many of these cancers respond to hormonal therapy and the presence of estrogen receptor (ERα) is associated with a more favorable response and short-term prognosis. Tamoxifen is a selective estrogen receptor modulator (SERM) that acts through ERα and is effective in the prevention and treatment of estrogen-dependent breast cancer. However, tamoxifen is estrogenic in the uterus and is associated with an increased incidence of endometrial cancers. In addition, advanced breast cancers invariably become resistant to tamoxifen. Here we report the crystal structure of a complex containing the estrogen receptor ligand-binding domain (ERα LBD) bound to a structurally similar compound, GW5638, which has clinical potential and exhibits no adverse effects in the uterus. Notably, tamoxifen resistant MCF-7 breast tumor explants still respond to GW5638. Although GW5638 induces a characteristic antagonist-ER LBD structure, it elicits a distinct conformation in the carboxyl-terminal activation (AF-2) helix (H12) through direct interactions with the N-terminus of H12, which has not been observed in any of the previously solved nuclear receptor LBD structures. Similar to 4-hydroxytamoxifen (OHT), GW5638 induces an autoinhibitory conformation of ER that prevents the binding of coactivators. However, in contrast to OHT, GW5638 repositions hydrophobic residues in H12, thereby increasing the exposed hydrophobic surface of ER, which correlates with a significant degradation of ERa in MCF-7 cells. Thus, the GW5638-ERα LBD structure reveals a novel mode of SERM-mediated ER antagonism in which the stability of ER is decreased through an altered position of H12. This dual mechanism may account for the ability of GW5638 to inhibit tamoxifen-resistant breast tumor explants.

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APPENDIX 3. Era of Hope 2005 poster abstract

STRUCTURAL BASIS FOR A NOVEL MODE OF SERM-MEDIATED ER ANTAGONISM

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Breast cancers affect one in eight women in the United States. Many of these cancers respond to hormonal therapy and the presence of estrogen receptor (ERalpha) is associated with a more favorable response and short-term prognosis. Tamoxifen is part of a growing family of molecules called selective estrogen receptor modulators (SERMs) that can behave as agonists or antagonists in different tissue and environmental contexts. It has been widely used for breast cancer treatment and shows considerable potential as a preventive agent. Unfortunately, advanced breast cancers that initially respond well to tamoxifen eventually become refractory to this compound. Its uterotrophic activity also restricts its utility in a prevention setting. We report the crystal structure of the estrogen receptor alpha (ERalpha) ligand-binding domain (LBD) bound to the structurally similar compound GW5638, which has therapeutic potential and does not stimulate the uterus. Like tamoxifen, GW5638 relocates the carboxy-terminal helix (H12) to the known coactivator-docking site in the ERalpha LBD. However, GW5638 repositions residues in H12 through specific contacts with the N-terminus of this helix. In contrast to tamoxifen, the resulting increase in exposed hydrophobic surface of ERalpha LBD correlates with a significant degradation of ERalpha in MCF-7 cells. Thus, the GW5638-ERalpha LBD structure reveals a unique mode of SERM-mediated ER antagonism, in which the stability of ERalpha is decreased through an altered position of H12. This dual mechanism of antagonism may explain why GW5638 can inhibit tamoxifen-resistant breast tumors.

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